ARGENE

ENTEROVIRUS R-gene®

REF 69-005

REF 69-005B

CE

IVD

COMPOSITION

REF 69-005	DNA/RNA EXTRACTION KIT	Ref.: 67-020
	ENTEROVIRUS R-gene® - Real-Time PCR kit	Ref.: 69-005B

ARGENE

1. Presentation of the kit

- Members of the Picornaviridae family, enteroviruses are single-strand RNA viruses, split into 4 families (Enterovirus A, B, C and D). More than100 serotypes have been described, of which 68 are currently recognized in the 2005 international classification (*8th report of the International Committee on the Taxonomy of Viruses. 2005*). In temperate climates, Enterovirus infections occur mostly seasonal (May to October) and are particularly common infections in children and adolescents. Enteroviruses are leading causes of viral meningitis. The clinical criteria of meningitis caused by an Enterovirus infection can not be discriminated from those caused by other infectious agents (e.g. Mumps virus or Herpes Simplex virus). Enteroviruses are also associated with cardiac, respiratory, cutaneous mucosa or neonatal pathologies. Polioviruses are responsible for acute anterior poliomyelitis. This disease is currently being eradicated worldwide.
- The standard method for the diagnosis of Enteroviruses is isolation by cell culture followed, if necessary, by a sero-neutralisation typing. However, this traditional method sometimes lacks sensitivity for certain serotypes (Coxsackievirus A and enterovirus 68 to 71). Currently, the method of choice for the diagnosis of neuro-meningitis infections is molecular diagnosis on CSF samples. Much faster than conventional PCR methods, Real time PCR enables a rapid and sensitive identification of all the serotypes, allowing clinicians to cut off unnecessary treatment and useless examinations.
- The ENTEROVIRUS R-gene® kit enables the generic detection of Enteroviruses by amplification of the highly conserved 5' non-coding region, by means of Real Time PCR amplification on CSF samples, stool samples, respiratory specimens or culture cells.
- This kit enables the detection of the following serotypes:
  - Enterovirus A: Coxsackievirus A4, A6 to A8, A10, A14, A16, A16V Enterovirus 71, 76
  - Enterovirus B: Coxsackievirus A9, B1 to B6, Echovirus 1 to 7, 9, 11 to 21, 24 to 27, 29 to 33, Enterovirus 69, 74, 75, 77, 78, 93
  - Enterovirus C: Coxsackievirus A11, A13, A17, A20, A21, A24, A24V Poliovirus 1, 2, 3
  - Enterovirus D: Enterovirus 68, 70, 94.
- The ENTEROVIRUS R-gene® kit does not detect Parechovirus 1, 2, 3 and Rhinovirus 9, 39 and 89. Note that Rhinovirus 14,17 and 22 are detected by this kit.
- The sample analysis can be simultaneously run for the other viruses: HSV1 HSV2 VZV R-gene® (Réf.: 69-004) and Parechovirus (Ref.: 71-020) with the program ENTEROVIRUS R-gene®.

2. Intended use

- The ENTEROVIRUS R-gene® kit allows rapid detection of enterovirus genomes using the Real Time PCR method on suitable samples (CSF, Throat, nasopharyngeal secretions, stool, cell culture).
- It is absolutely necessary to compare results obtained with ENTEROVIRUS R-gene® kit to other diagnostic investigation methods in order to define patient viral status.

3. Principle of the test

3.1. RNA purification:

- The following RNA extraction method are validated with ENTEROVIRUS R-gene® kit, ref.: 69-005B:
  - NucliSENS® easy MAG®
  - MagNA Pure Compact Instrument
  - Versant® kPCR Molecular System SP
  - QIAcube
  - EZ1 Advanced / EZ1 Advanced XL
  - DNA/RNA EXTRACTION KIT
  - QIAamp® MinElute Virus Spin
  - QIAamp® Viral RNA Mini kit
- Both the target RNA contained in the sample and in the extraction + inhibition control (**IC1**) are extracted using one of the extraction methods above.
- The technique used by the DNA/RNA EXTRACTION KIT (Ref.: 67-020) associates the selective binding properties of silica gels with a micro centrifugation speed. The sample and internal control (**IC1**) are first lysed in highly denatured conditions in order to inactivate the RNases and obtain free viral nucleic acids. The nucleic acids are taken up in a selected buffer to optimize their ability to bind to the membrane. By using a silica column, once the nucleic acids are bound, the sample can effectively be washed to eliminate contaminants. The elution stage, using a buffer which does not contain RNase, produces purified nucleic acids for amplification.

ARGENE

3.2. Real time amplification and detection:

- The principle of the real-time detection lies within the use of the 5' nuclease technology (Patents N°5210015, 5487972), also called TaqMan® probes. The ready-to-use amplification mixture includes: primers, dNTPs, amplification buffer, Taq Polymerase, specific probes for Enterovirus and the specific primers and probes for the internal control (**IC1**) introduced during extraction (lysis).
- The following range of real time PCR platforms are validated with ENTEROVIRUS R-gene® kit, ref.: 69-005B:
  - LightCycler®
  - Applied Biosystems
  - Stratagene®, Agilent or Versant® kPCR Molecular System AD
  - SmartCycler® 2.0
  - Rotor-Gene®
  - Dx Real-Time System (Bio-Rad)
- The reverse transcription and amplification steps are performed in one step. The primers used enable the specific sequences located in the 5th non-coding region of the genome to be amplified. In one single amplification, they enable the many enterovirus serotypes to be shown. The amplified fragment size is 146 bp.
- A positive control enables the experiment to be properly validated. This positive control is amplified with the same primers as the viral RNA of the Enterovirus potentially present in patient samples.
- An extraction and inhibition control (**IC1**) is included in the ENTEROVIRUS R-gene® kit (Ref: 69-005B) in order to check, starting from the lysis step if the sample has been well extracted and to verify the presence of amplification inhibitors in the sample.

WARNING: **The described performances can only be guaranteed for the recommended extraction systems and PCR instruments.**

4. Content of the kit and storage

4.1. « DNA/RNA Extraction Kit » 67-020

- Number of extractions per kit: 50

A	QIAamp® MinElute column	5 x 10
B	Collection tubes (2 mL)	4 x 50
C	AL buffer Xn – HARMFUL	12 mL
D	AW1 buffer (concentrated) Xn- HARMFUL	19 mL
E	AW2 buffer (concentrated)	13 mL
F	AVE buffer	5 x 2 mL
G	Carrier RNA	310 µg
H	QIAGEN protease Xn- HARMFUL	1 vial

Package Insert : 1 Package Insert provided in the kit or downloadable from www.biomerieux.com/techlib

- This kit can be stored before and after first opening (+2°C/+8°C) until the expiry date written on th e box, except for the carrier RNA (**G**) which is stored at -18/-22°c after reconstitution.

4.2. Real-time PCR kit Enterovirus R-gene® 69-005B

- Number of tests: 90

W0	Water for extraction (Molecular grade)	2 x 1.8 mL
IC1	Internal Control 1	1 mL
RT	Reverse transcriptase Omniscript® (ready to use)	35 µL
R9	Enterovirus and IC1 Amplification premix	3 x 0.450 mL
PC9	Enterovirus positive Control	300 µL

Package Insert : 1 Package Insert provided in the kit or downloadable from www.biomerieux.com/techlib

- Keep the kit 69-005B before and after first opening frozen at -18°C/-22°C, in the dark, until expiry date printed on the box.
- Before and after opening the kit (ref. : 69-005B), Positive control (**PC9**), Internal control 1 (**IC1**) and reagent (**W0**) must be stored in the extraction room at -18°C/-22°C.
  The reagents **R9** and **RT** must be stored in the room reserved for the preparation of the premix at -18°C/-22°C.
- Each amplification premix (**R9**) cannot undergo more than 11 freezing/defrosting cycles.
- Return the amplification premix (**R9**) and the positive control (**PC9**) at -18°C/-22°C immediately after use.

ARGENE

5. Material and reagents required but not supplied

5.1. For sample extraction:

5.1.1. With DNA/RNA Extraction kit (Ref.: 67-020)

- Ethanol 96-100%
- Centrifuge (6 000 g – 12 000 g)
- Vortex
- Test tubes (1,5 mL, 2 mL)
- Water bath +56°C
- Sterile micropipettes with plugged (aerosol barrier) tips or positive displacement tips
- Single use latex or similar gloves

5.1.2. Other extraction methods validated

- Follow the manufacturer's instructions

5.2. For Enterovirus R-gene® kit, ref.: 69-005B:

- Micropipettes with plugged (aerosol barrier) tips or positive displacement tips;
- Thermocyclers validated with ENTEROVIRUS R-gene® kit.
- LC Carrousel Centrifuge for LightCycler® or benchtop microcentrifuge convenient for 2 mL reaction tubes or plate centrifuge for Applied Biosystems, Dx Real-Time System and Stratagene®, Agilent or Versant™ kPCR Molecular System AD;
- Single use latex or similar gloves;
- Capillaries, tubes, microplates for real time PCR platforms validated for ENTEROVIRUS R-gene®;
- Cooling block suitable for the thermocycler of choice;
- UV Light;
- Workstation or plexiglass screen for samples and premix distribution;
- Colour Compensation r-gene® (Argene ref.: 71-103) for result interpretation on LightCycler® 2.0;

6. Reagent reconstitution

ONLY reconstitute reagents supplied with the DNA/RNA EXTRACTION kit (ref.: 67-020).

6.1. Protease stock solution preparation

- Add 1.4 mL of AVE buffer (**F**) to the 24 mg of lyophilized protease (**H**).
- Store aliquots at -18°C/-22°C (repeated freezing a nd thawing must be avoided).

6.2. AL buffer (C) preparation

- AL buffer (**C**) is stable when stored tightly closed at +2°C/+8°C .
- Mix AL buffer (**C**) thoroughly by shaking before use.
- Do not store protease mixed with AL buffer (**C**).

6.3. AW1 buffer (D) preparation

- AW1 (**D**) buffer is stable when stored tightly closed at +2°C/+8°C.
- AW1 buffer (**D**) is supplied as a concentrate. Prior to first time use, add a volume of 25 mL of ethanol (96-100%) to the 19 mL of concentrated buffer.

6.4. AW2 buffer (E) preparation

- AW2 (E) buffer is stable when stored tightly closed at +2°C/+8°C.
- AW2 buffer (**E**) is supplied as a concentrate. Prior to first time use, add a volume of 30 mL of ethanol (96-100%) to the 13 mL of concentrated buffer.

6.5. Carrier RNA (G) preparation

- Add 310 µL of buffer AVE (**F**) to one tube of lyophilized carrier RNA (**G**). Dissolve Carrier RNA thoroughly. Mix thoroughly before using for the first time.
- This stock solution of carrier RNA is stored aliquoted at -18°C/-22°C (avoid freeze/thaw cycles).

ARGENE

7. Warnings and precautions

7.1. Warnings and precautions for molecular biology

- The kit must be handled by a qualified staff member, in accordance with Good Laboratory Practice and handling instructions for molecular biology.
- Amplification procedures require the following precautions to avoid the risk of sample contamination:
  - Use separate working places for sample preparation and amplification reactions. Movement in the laboratory must be in one direction only from the reagent preparation area to the amplification area. Allocate a set of lab coats and pipettes to each area. Never introduce an amplified product in reagent and/or sample preparation areas.
  - Samples must be exclusively reserved for this analysis.
  - Samples must be prepared in a biological safety cabinet. Tubes from different specimens must never be opened at the same time.
  - Pipettes used to handle samples must be reserved for this purpose only. These pipettes must be positive displacement pipettes or pipettes equipped with sterile filter tips.
  - The pipettes used to aliquot reagents must be reserved for this purpose only. The necessary reagents for amplification must be aliquoted and used for one single experiment.
  - The reagents must be fully defrosted to room temperature before testing.

7.2. General warnings and precautions

- Handle and dispose all specimens and materials as potentially infectious. After use: material, reagents and waste must be handled as potentially infectious.
- Read all instructions before performing this assay.
- Respect expiration date (printed on the labels).
- Use only reagents provided with the kit.
- Do not substitute reagents from kits with different batch numbers or from other manufacturers.
- Never pipette by mouth.
- Do not smoke, eat or drink in dedicated work areas.

7.3. Reagent specific warning and precautions

- AL (**C**) buffer and AW1 buffer (**D**) contain guanidinium chloride (chaotropic salt).
  - R22: Harmful if swallowed.
  - R36/38: Irritating for eyes and skin.
  - S13: Keep away from food, drinks and animal feeding supplies.
  - S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
  - S36: Wear suitable protective clothing.
  - S46: If swallowed, seek medical advice immediately and show the container or label.

  This component must not be used with disinfecting agents that contain bleach.

- AW2 buffer (**E**) contains 0.04% sodium azide as preservative.

- Protease (**H**) contains subtilisin
  - R37/38: Irritating to respiratory system and skin.
  - R41: Risk of serious damage to eyes.
  - R42: May cause sensitisation by inhalation.
  - S22: Do not breathe dust.
  - S24: Avoid contact with skin.
  - S26: In case of contact with eyes, rinse immediately with plenty of water and seek medical advice.
  - S36/37/39: Wear suitable protective clothing, gloves and eye/face protection.
  - S46: If swallowed, seek medical advice immediately and show the container or label.

- Avoid contact between the reagents and the skin. Wash immediately with copious amounts of water if contact occurs. Wear gloves when handling the reagents.

ARGENE

8. Controls

530 nm = the reading channel "FAM" or "Green" or other depending on the real-time PCR platforms. For reasons of simplification, only "530 nm" is used.

560 nm = the reading channel "VIC", "Hex" or other according to the real-time PCR platforms. For reasons of simplification, only "560 nm" is used.

CT = Crossing Threshold for most real-time PCR platforms or CP (Crossing Point) for devices in the LightCycler® range. For reasons of simplification, only CT is used.

The order for adding samples and controls must be followed (see chapter « Amplification preparation »).

8.1. Extraction + Inhibition controls

8.1.1. Sample extraction+inhibition control

- This control consists of the internal control (IC1) to be added to the samples, extracted and amplified, in order to control the effectiveness of extraction and detect the possible presence of inhibitors.
- Signal reading is 560 nm.

8.1.2. Reference extraction+inhibition control

- This control consists of the internal control (IC1) added to the negative extraction control (**W0**), extracted and amplified at the same time as the samples to obtain a reference (**IC1W0**). It must be compared with the extraction+inhibition control of all samples (**IC1sample**).
- Signal reading is 560 nm.

==> The comparison of the CT (Crossing Threshold) value at 560 nm of both controls (**IC1W0** and **IC1sample**) will show any amplification inhibition and the success of the extraction step.

8.2. Negative extraction + amplification control (IC1W0)

- This is the same tube as described in 8.1.2 (reference extraction + inhibition control) but when the reading is done at 530 nm then it is a negative control that allows the absence of contamination to be verified at extraction and amplification.
- Signal reading is 530 nm.

8.3. Positive amplification control (PC9)

- This control (PC9) contains a plasmid that hybridizes specifically with the *enterovirus* primers contained in the reaction mixture R9.
- This control shows any possible contamination during the amplification step.
- Signal reading is 530 nm.

9. Sample treatment and transport

Samples are withdrawn and transfer under the laboratory instructions.

WARNING: **Avoid diluting the samples and/or extracts in mediums containing phosphate (mediums such as transport, supernatant culture, dilutions pre and post extraction in PBS). This could decrease extraction efficiency.**

9.1. Sample transport

- For samples to be transported check your local legislation for hazardous and infectious material transport.
- Samples must be transported and treated by the laboratory in the shortest possible time (preferably within 24 hours).

9.2. Sample treatment

9.2.1. CSF samples

- CSF is collected in dry tubes following classical conditions of lumbar puncture.
- CSF sent in dry ice must be stored at -18℃/-22℃ or preferentially -78℃/-82℃.

9.2.2. Stool dilution preparation

a) For an automatic extraction with NucliSENS® easyMAG® :

- Add a large lens of stools at 800 µL of NucliSENS lysis pad.
- Vortex vigorously.
- Incubate for 10 minutes at room temperature (+18/+25℃).
- Centrifuge for 30 minutes at 3000g.

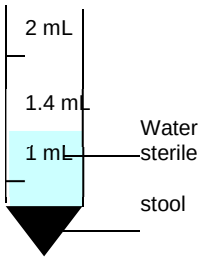
- Transfer the supernatant into the control well of the corresponding easyMAG® loader.

ARGENE

b) For other extractions:

- Make a stool suspension of 10 to 50% in 1 mL of water sterile in the following way:
  - In a 2 mL marked centrifuge tube pipet 1 mL of water sterile. Add the stool specimen described to obtain a suspension of which the total volume may vary from 1.1 mL (stool suspension at 10%) to 1.5 mL (stool suspension at 50%).
  - Mix well.

Example: Stool suspension at 40%



  - Centrifuge at 6000g for 30 minutes. Collect the supernatant.
  - Centrifuge at 12000g for 15 minutes. Collect the supernatant.
  - This supernatant constitutes the sample to extract, it can be preserved at **-78/-82 °C**.

9.2.3. Respiratory samples preparation:

- The respiratory specimens (solid or swab samples) should be suspended again, if necessary, in a small volume of normal saline solution and centrifuged at 4000-6000 rpm for 6 minutes before carrying out the test.
- In the event of extracting using NucliSENS® easyMAG® automated system, sample + **IC1** pre-treatment with Proteinase K is necessary. In this case, add 10 µL of Proteinase K to 20 mg/mL and incubate for 15 minutes at 56°C.

10. Sample extraction protocol

WARNING: **Before starting the extraction procedure, make sure samples and reagent IC1 have been homogenized**

Avoid diluting the samples and/or extracts in mediums containing phosphate (mediums such as transport, supernatant culture, dilutions pre and post extraction in PBS). This could decrease extraction efficiency.

In the room reserved for sample extraction

10.1. With « DNA/RNA Extraction Kit » (Ref.: 67-020 + IC1 + W0)

- Equilibrate samples, **IC1** and **W0** to room temperature +18°C/+25°C.
- Equilibrate AVE buffer (**F**) to room temperature +18°C/+25°C.
- Check that AL (**C**), AW1 (**D**), AW2 (**F**) and carrier RNA (**G**) have been prepared according to the instructions given in section "Reagent reconstitution". Use one aliquot per run.

Note 1: All centrifugation steps are carried out at room temperature.
Note 2: Stool should be previously diluted in 10 % proportion in sterile water (see section *« Stool dilution preparation »).*

10.1.1. Extemporaneous preparation of the mixture of AL Buffer (C) + carrier RNA (G):

- Calculate the volume of AL buffer to pipet:
  - **n** = the number of tests (samples + **W0**)
  - Pipet **n** x 0.22 mL of AL buffer.
- Add carrier RNA at 28 µL/mL of AL buffer according to the following table:

Tests Nb **n**	Buffer Vol. AL (mL)	Carrier RNA Vol. (µL)	Tests Nb **n**	Buffer Vol. AL (mL)	Carrier RNA Vol. (µL)	Tests Nb **n**	Buffer Vol. AL (mL)	Carrier RNA Vol. (µL)
2	0.44	12.3	10	2.20	61.6	18	3.96	110.9
3	0.66	18.5	11	2.42	67.8	19	4.18	117.0
4	0.88	24.6	12	2.64	73.9	20	4.40	123.2
5	1.10	30.8	13	2.86	80.1	21	4.62	129.4
6	1.32	37.0	14	3.08	86.3	22	4.84	135.5
7	1.54	43.1	15	3.30	92.4	23	5.06	141.7
8	1.76	49.3	16	3.52	98.6	24	5.28	147.8
9	1.98	55.4	17	3.74	104.7	25	5.50	154.0

Example for 4 samples to analyse in a series :

***n** = 4+1 = 5 ;*
Volume of AL buffer to pipet = 1.1 mL ;
Volume of carrier RNA to be added = 30.8 µL

ARGENE

10.1.2. Lysis step

- Prepare and identify (on the lid) an equal number of 1.5 mL microcentrifuge tubes to samples being analyzed. Add **ONE** tube dedicated to the extraction of the mix (**W0**+IC1).
- Add 25 µL of protease (**H**).
- Pipet 200 µL of prepared AL/RNA carrier in each tube.
- Add 10 µL of internal control **IC1.**

> *To use HSV1, HSV2, VZV R-gene® and Enterovirus R-gene® kits simultaneously, add 10 µL IC2 + 10 µL IC1 to the sample.*

- Add 200 µL of **W0** in the tube identified for the mix (**W0**+IC1).
- Add 200 µL sample and mix by pulse-vortexing for 15 seconds. To ensure efficient lysis, it is essential that the sample is mixed thoroughly to yield a homogeneous solution.
- If the volume is less than 200 µL, add sterile water to adjust final volume to 200 µL.
- Incubate at +56°C for 15 minutes.
- Briefly centrifuge the 1.5 mL microcentrifuge tube to remove any droplets from the inside of the lid.

10.1.3. Loading step

- Add 250 µL of 96-100% ethanol to the sample, and mix by pulse-vortexing for 15 seconds. Only ethanol should be used since other alcohols may result in reduced RNA yield and purity.
- Incubate at room temperature for 5 minutes.
- Briefly centrifuge the 2 mL microcentrifuge tube to remove any droplets from inside of the lid.
- Prepare and identify the same number of spin columns as samples to be tested.
- Carefully apply the sample volume to the spin column (in a 2 mL collection tube) without wetting the rim.
- Close the cap, and centrifuge at 6000xg for 1minute.
  Note: Each column must be closed during centrifugation in order to avoid cross contaminations between samples.
- If there is solution remaining on the column membrane, centrifuge it again until the solution goes through the membrane.

10.1.4. Washing step

- Carefully open the spin column and add 500 µL of buffer AW1 (**D**). Close the cap and centrifuge at 6 000 g for one minute. Place the spin column in a clean 2 mL collection tube (provided), and discard the tube containing the filtrate.
- Carefully open the spin column and add 500 µL of buffer AW2 (**F**). Close the cap and centrifuge at 6 000 g. using a microcentrifuge for 1 minute. Place the spin column in a clean 2 mL microcentrifuge tube (not provided) and discard the old collection tube with the filtrate.
- An intermediate stage must be added by placing the columns over a clean 2 mL tube and centrifuge for 3 minutes at high speed (12 000 g) before eluting into a new 2 mL tube. This stage eliminates all risk of ethanol rising through the column during centrifuge deceleration or when transferring the columns out of the rotor.

10.1.5. Elution step

- *All samples except stool samples :*
  Add 50 µL of buffer AVE (**F**) equilibrated to room temperature at the center of the column. Close the cap and incubate for 5 minutes at room temperature. Centrifuge at top speed (12 000 g) for 1 minute.
- *Stool samples:*
  Elute once with 60 µL of AVE buffer (F). Incubate for 5 minutes at room temperature, centrifuge at 20 000 g for 1 minute.

- The eluate constitutes the extracted sample. It is used directly in the next stage or it can be frozen at -78°C/-82°C for a few days to a few weeks.

ARGENE

10.2. With extraction instruments and/or kits validated with Enterovirus R-gene®

- These extraction instruments must be regularly maintained as recommended by the manufacturer by a qualified and trained staff.

Instruments	Kit	Sample + IC1 Volumes	Sample type	Protocol	Elution volume
	QIAamp® Viral RNA Mini kit Ref. : 52 904 / 52 906	140 µL of sample + 10 µL IC1	CSF		40 µL
			Respiratory samples Stool		60 µL
QIAcube QIAGEN Ref. : 9001292 / 9001293			CSF	Purification of viral RNA from cell-free body fluids	50 µL
			Respiratory samples Stool		60 µL
EZ1 Advanced / EZ1 Advanced XL	EZ1 Virus Mini kit	200 µL of sample + 10 µL IC1	CSF	EZ1 Virus Card v2.0	60 µL
MagNA Pure Compact® Roche Diagnostics Ref.: 03 731 146 001	MagNA Pure Compact Nucleic Acid Isolation Kit I Réf. : 03 730 964 001 (32 isolations)		CSF Respiratory samples	Total_NA_Plasma_100_400	50 µL
	MagNa Pure Compact RNA Isolation Kit Réf. : 04 802 993 001		CSF Respiratory samples	RNA_Blood_V3_2	50 µL
NucliSENS® easyMAG® bioMerieux Ref. 280110	NucliSens® magnetic extraction reagents		CSF Respiratory samples	Generic	50 µL
				Specific A + matrix	70 µL
			Stool	Specific A + 100 µL of silica	50 µL
Versant® kPCR Molecular System SP SIEMENS Ref. 06635740	Sample Preparation 1.0	400 µL of sample + 10 µL IC1* (extraction 250 µL)	CSF	Sample Preparation Protocol 5	65µL (eluate de 50µL)

* To extract samples of an initial volume higher than 400µL, add the following amounts of IC1 reagent:
For 1 initial sample amount, add 1/40th the amount of IC1 reagent (for example: to extract a 600µL sample, add 15µL of IC1 reagent).

- For extraction with the NucliSENS® easyMAG® automatic system:
  - For CSF: The choice of protocol must be appropriate to the laboratory organization.
    - The Generic protocol, with elution at 50 µL is used to search for herpesviruses also in the extract, with good sensitivity.
    - The Specific protocol A + 100 µL of plasma matrix, with elution in 70 µL is less sensitive in CSF for which the Generic protocol is recommended.
  - For respiratory samples and extraction with the NucliSENS® easyMAG® automatic system, pre-processing of (samples + IC1) with proteinase K is necessary. In this case, add 10 µL of Proteinase K at 20 mg/mL and incubate for 15 minutes at 56°C.

To use HSV1, HSV2, VZV R-gene® and Enterovirus R-gene® kits simultaneously, add 10 µL IC2 + 10 µL IC1 to the sample.

ARGENE

11. Detection and Real-time PCR quantification protocol

Note: With a view to simplifying the instructions, the device dedicated to holding the amplification reaction mix is referred to as a "tube".

- The product to be amplified corresponds to the extracted DNA obtained with the extraction methods recommended with the ENTEROVIRUS R-gene® kit.
- Schedule:

  - 1 tube per tested sample;
  - 1 tube for reference extraction + inhibition control (**IC1W0**) also used as negative control for extraction and amplification;
  - 1 tube for the positive amplification control (**PC9**).

- Use the transparent plates (ref.: HSP9601) with the optical stoppers (ref.: TCS0803) for the Dx Real-Time System amplification device).

11.1. Thermocycler programming:

- Whatever the real time PCR platform used, the amplification program remains the same. The amplification program is described in the table below:

Steps		Time	Temperature	Cycles	Fluorescence acquisition:						
					LC1	LC2, LC480	Applied Biosystems	Rotor-Gene®	SC2	Stratagene Versant™ kPCR Molecular System AD	Dx Real-Time System
Reverse Transcription		30 min.	50℃	1	-	-	-	-	-	-	-
Taq Polymerase Activation		15 min.	95℃	1	-	-	-	-	-	-	-
Amplification	**Denaturation**	10 sec.	95℃	45	-	-	-	-	-	-	-
	Hybridization	40 sec.	60℃		**530**	**530 560**	**FAM VIC**	**Green Yellow**	**FAM Cy3**	**FAM HEX**	
					End of the hybridization						
	Elongation	25 sec.	72 ℃		-	-	-	-	-	-	-

To simultaneously detect the HSV-1, HSV-2, VZV viruses and/or enteroviruses, use the amplification program described above.

Note 1: Temperature transition rate/slope is pre-defined until 20℃/sec or 100%.
Note 2 : On LightCycler® instruments, add a cooling step : 30 sec / 40℃ / 1 cycle at the end of the PCR.
Note 3 : On LightCycler®, adjust the «SEEK TEMPERATURE» parameter to 50℃ when programming.
Note 4 : On LightCycler® 2, using a colour compensation file is ESSENTIAL for interpreting results. Check that it has been created and saved in the management software for LightCycler® 2.0 by using the r-gene® Colour Compensation kit (Argene ref.: 71-103).
Note 5 : On LightCycler® 480, there are two optical systems: only "System II" is compatible with the ENTEROVIRUS R-gene® kit. "System II" includes automatic colour compensation in its software.
Note 6 : On Applied Biosystems select « NONE » in « PASSIVE REFERENCE » when programming.
Note 7 : On Rotor-gene®, calibrate the signal by clicking on "GAIN OPTIMIZATION".
Note 8: On Stratagene®, Agilent or Versant® kPCR Molecular System AD select "NONE" in "REFERENCE DYE".

Details on programming according to device type are available on request.

11.2. Amplification preparation

In the room reserved for Amplification

- Before starting the experiment:
  - The reagents must be fully defrosted at room temperature before testing.
  - Mix each reagent (to a vortex for 2 seconds or through successive pipetting) and centrifuge briefly.
  - Make sure the cooling block was decontaminated by exposure under U.V. light for 30 min.
  - Make sure the cooling block was correctly pre-cooled at +2℃/ +8℃.

WARNING :
- In order to keep contamination to a minimum, close the tubes as you go along.
- Replace the amplification premix (R9) and the positive control (PC9) at -18℃/-22℃ immediately after use.
- Each premix cannot undergo more than 11 freezing/defrosting cycles.
- Number « n » is a multiple corresponding to the number of sample to test + 2 controls. It is used to calculate the reaction volumes.
n = number of samples + 2

Example for 7 samples to be analysed in one run : **n** = 7 + 2 = 9

ARGENE

11.2.1. Amplification premix preparation:

- Pipet (**n**+1) x 15 µL of amplification premix (**R9**) and place in an appropriate tube (0.2 mL – 0.5 mL).
- Add (**n**+1) x 0.1 µL of **RT**.

Example for 7 samples (n=9) : Pipet 1 µL of RT + 150 µL of R9 and dispense it in one appropriate tube.

11.2.2. Addition of samples and controls in premix solution:

- Collect 15 µL of the amplification premix by gently homogenizing with the pipette in order to distribute the same volume in all capillaries and distribute 15 µL of amplification premix in all tubes.

The order for adding samples/reagents as shown below must be followed (see section "Amplification preparation"):

- Add 10 µL of each extracted sample in the corresponding tube.
- Add 10 µL of extracted mix **IC1+W0** in the corresponding tube. This tube is the **IC1W0** control (see section "Controls").
- Add 10 µL of **PC9** in the corresponding tube. This tube is the positive amplification control (see section "Controls").
- Centrifuge the tubes with the corresponding device and transfer them in the thermocycler.

11.3. Running the Enterovirus R-gene® program:

- Start the amplification program (stored according to the instructions described in section "Thermocycler programming").
- Define samples and controls.

12. Data Analysis

The detail of the exploitation of the results by type of apparatus is available upon demand.

***530 nm** = the reading channel "FAM" or "Green" or other depending on the real-time PCR platforms. For reasons of simplification, only "**530 nm**" is used.*

***560 nm** = the reading channel "VIC", "Hex" or other according to the real-time PCR platforms. For reasons of simplification, only "**560 nm**" is used.*

12.1. With LightCycler® 1.0

- Apply the FIT POINTS method in ARITHMETIC mode using 2 data points.
- Move the threshold line (red horizontal cursor line) to a position where it crosses all fluorescence curves of samples in their linear part, above the baseline noise.

  If the position of the crossing line is not sufficient for crossing all the sample curves in their linear part, repeat the step as frequently as necessary to obtain the CP for each sample.
- For each positive sample, a CROSSING POINT (CP) is calculated.

12.2. With LightCycler® 2.0

- Analysis of viral target is carried out in ABSOLUTE QUANTIFICATION mode at 530NM.
- Analysis of extraction + inhibition control is carried out in ABSOLUTE QUANTIFICATION mode at 560NM after activation of colour compensation file (COLOUR COMPENSATION tab.) (Argene **Colour Compensation r-gene®** ref: 71-103).
- Apply FIT POINTS method.
- Move the threshold line (red horizontal cursor line) to a position where it crosses all fluorescence curves of the samples in their linear part, above the baseline noise.

  If the position of the crossing line is not sufficient for crossing all the sample curves in their linear part, repeat the step as frequently as necessary to obtain the CP for each sample.
- For each positive sample, a CROSSING POINT (CP) is calculated at 530nm.
- The extraction+inhibition controls are analysed by comparing the calculated CP value for each extraction + inhibition control (**IC1sample**) to the CP value obtained with the reference extraction + inhibition test (**IC1W0**) at 560 nm.

12.3. With LightCycler® 480 (System II)

- Switch on the LC480 (System II) FAM – HEX automatic compensation.
- Analysis of the viral target is carried out in the ABSOLUTE QUANTIFICATION mode at 530nm (FAM).
- Analysis of the extraction + inhibition control is carried out in the ABSOLUTE QUANTIFICATION mode at 560nm.
- For each positive sample, a CROSSING POINT (CP) is calculated at 530nm.
- The extraction+inhibition controls are analysed by comparing the calculated CP value for each extraction + inhibition control (**IC1sample**) to the CP value obtained with the reference extraction + inhibition test (**IC1W0**) at 560 nm.

ARGENE

12.4. With SmartCycler® 2.0

- Analysis of the viral target is carried out in the **FAM** mode at **530nm**.
- Analysis of the extraction + inhibition control is carried out in the **CY3** mode at **560nm**.
- For each positive sample, a **CROSSING THRESHOLD** (CT) is calculated at **530nm** (**FAM CT**).
- The extraction + inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition controls (**IC1sample**) to the CT value obtained with the reference extraction + inhibition test (**IC1W0**) at **560 nm** (**CY3 CT**).

12.5. With Applied Biosystems (including StepOne® et Applied Biosystems Fast)

- Check if **NONE** is selected in the field **PASSIVE REFERENCE** because the **R9** premix does not contain passive reference fluorochrome.
- Analysis of samples is carried out after having selected the **FAM R-GENE** detector in the **DETECTOR** field.
- Manually adjust the *threshold line* manually to a position where it crosses all fluorescence curves of the samples in their linear part. This step is performed to identify the positive samples which correspond to a calculated CT value. Negative samples are defined as **UNDETERMINED** displayed in the **CT** column by the SDS software.
- Analysis of extraction + inhibition controls (**IC1sample** and **IC1W0**) is carried out in the same way after having selected the **VIC R-GENE** detector in the **DETECTOR** field.
- The extraction+inhibition controls are analysed by comparing the calculated CT value for each extraction + inhibition control (**IC1sample**) at CT value obtained with the reference extraction + inhibition test (**IC1W0**) at **560 nm**.

12.6. With ROTOR-GENE®

- Analysis of the viral target is carried out in the **CYCLING A GREEN** mode at **530nm**.
- Analysis of the extraction + inhibition control is carried out in the **CYCLING A YELLOW** mode at **560nm**.
- Manually adjust the threshold line above initial noise band in the **LINEAR SCALE** mode after selecting **DYNAMIC TUBES** and **SLOPE CORRECT** to a position where it crosses all fluorescence curves of the samples in their linear part. This step is performed to identify the positive samples which correspond to a calculated **CROSSING THRESHOLD** (CT) value at **530 nm**.
- For each positive sample, a **CROSSING THRESHOLD** (CT) is calculated at **530nm**.
- The extraction+inhibition controls are analysed by comparing the calculated CT and the final fluorescence value for each extraction + inhibition control (**IC1sample**) at CT and the final fluorescence value obtained with the reference extraction + inhibition test (**IC1W0**) at **560 nm**.

12.7. Analysis of data on Stratagene®, Agilent or Versant™ kPCR Molecular System AD

- Make sure that **none** is selected in the **Reference Dye** field, because the premix **R9** does not contain passive reference fluorochrome.
- The viral target is analysed by pressing the **ANALYSIS** button then the **RESULTS** tab.
- The samples are analysed after selecting the **FAM** detector in the **ASSAYS SHOWN** field.
- Adjust the threshold line manually so that it cuts every amplification curve in its linear part. This stage determines the positive samples for which a CT is calculated. The negative samples are marked **NO CT** in the Ct column.
- The extraction + inhibition tests (**IC1sample** and **IC1W0**) are analysed in the same way after selecting the **HEX** detector in the **ASSAYS SHOWN** field.

12.8. Analysis of data on Dx Real-Time System.

- Analysis of the viral target: select the **QUANTITATION** tab leaving the **FAM** button checked.
- If necessary, manually adjust the threshold line so as to cross each amplification curve at the end of the exponential phase.
  This step aims to identify positive samples for which a CT is calculated. Negative samples are indicated by **N/A** in the **CT** column.
- The analysis of the extraction + inhibition controls (**IC1sample** and **IC1W0**) are performed in the same way after selecting the **HEX** detector.

ARGENE

13. Validation and interpretation of results

13.1. Test validation:

WARNING: **The test is only valid if all following conditions are fulfilled. If this is not the case, all samples and controls must be tested again.**

CT = Crossing Threshold for most real-time PCR platforms or CP (Crossing Point) for devices in the LightCycler® range. For reasons of simplification, only CT is used.

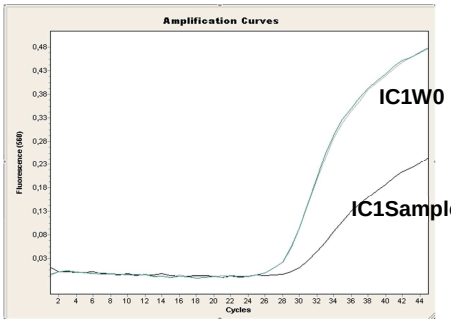
- 1st CONDITION: The negative extraction+amplification control **IC1W0** should not give a detectable signal (CT) at **530 nm**.
- 2nd CONDITION: The CT value of the reference extraction+inhibition control **IC1W0** must be equal or lower than 36 cycles at **560 nm**.
- 3d CONDITION: The CT value of the positive control (**PC9**) must be between 18 and 25 cycles at **530 nm**.
  - ⇨ If all conditions are fulfilled, the results obtained with the samples can be validated.

13.2. Interpretation of results

- Each sample must be analysed one by one.
- A positive sample displays a CT value at **530 nm**.
- If no CT value can be calculated at **530 nm**, the sample is considered as negative or its amplification is inhibited.
- However, in all cases, it is necessary to ensure there is no inhibition and that the extraction stage at **560 nm** was carried out correctly according to the factors described below.
- A strongly positive sample may generate inhibition. This has no effect on the analysis.

EXTRACTION and INHIBITION Control	CT[IC1sample] ≤ CT [IC1W0] + 3 cycles		CT[IC1sample] > CT [IC1W0] + 3 cycles	
	NON INHIBITED sample and correctly extracted		**INHIBITED** sample and/or badly extracted	
Sample	Calculated CT	Non calculated CT	Calculated CT	Non calculated CT
ENTEROVIRUS Detection	**POSITIVITY** validated	**NEGATIVITY** validated	**POSITIVITY** validated	**Negativity not validated** sample has to be extracted again

WARNING: **In case of a negative sample:**
If the slope of the curve generates a fall of final fluorescence (≥ 50%) compared to the final fluorescence of IC1W0 (see figure opposite) a weak inhibition is possible. We suggest extracting and to test the sample again.



IMPORTANT NOTE:
it is absolutely necessary to compare results obtained with ENTEROVIRUS R-gene® kit to other diagnostic investigation methods in order to define patient viral status.

The purchase of this product grants the purchaser rights under certain roche patents to use it solely for providing human in vitro diagnostic services. No general patent or other licence of any kind other than this specific right of use from purchase is granted hereby by Argene.

ARGENE

14. Trouble shooting

14.1. No signal or underestimated quantification in positive samples.

POSSIBLE CAUSES	RECOMMENDATIONS
The amplification premix has been defrosted too many times. The amplification premix has remained at room temperature for too long or has been defrosted at too high a temperature.	• Please refer to the "Content of the kit and storage" section. The premixes must not be defrosted more than 11 times. • Check that the amplification premixes and the positive control have been returned to -18/-22°C immediately after use. • Check that the amplification premixes have been defrosted at room temperature.
Failure to adhere to conditions for transport and storage of samples in the laboratory.	• Act in accordance with chapter «Sample treatment and transport» that defines the optimum conditions (temperature, time) for transport and storage.
Failure of storage conditions and expiry date of the Enterovirus R-gene®, Ref.: 69-005B.	• Follow instructions in section «Content of the kit and storage » regarding the storage of ENTEROVIRUS R-gene® at -18°C/-22°C in the dark.
Insufficient extraction step.	• Check if you carefully homogenized the samples before performing extraction. • Perform all washing steps and respect the incubation time when using «DNA/RNA EXTRACTION KIT ». Ref.: 67-020 (See section «With DNA/RNA Extraction Kit »). • Check if material and protocol used to extract samples correspond to material and protocol recommended for analysis with ENTEROVIRUS R-gene® Ref.: 69-005B (See section «With extraction instruments and/or kits validated with ENTEROVIRUS R-gene®»). • The kit's performance is only validated for " DNA/RNA EXTRACTION KIT" and extractions using automatic systems as described in chapter «With extraction instruments and/or kits validated with Enterovirus R-gene®». • Always perform preventive maintenance of workstations for automated extraction, and centrifuge systems, according to the manufacturer's recommendations.
Pipetting error.	• Check the calibration of your pipettes. • Check the distributed volume of reagents and samples: 15 µL of premix and 10 µL of sample. • Carefully homogenize reagents and samples before their distribution in capillaries/wells/tubes.
Programming error.	• Check all programming data (detection channel, mode, number of cycles, temperature and time). • Check all the steps regarding the entry of the samples.
Problem in amplification step	• Check the performances of the real time PCR platform as recommended by the manufacturer. • Check the attachment of the locking ring of the Rotor-Gene® carrousel. • Always perform preventive maintenance of real time PCR platform, and centrifuge, systems according to the manufacturer's recommendations.
Error in data analysis	• Check the baseline adjustment.
Error in interpretation results.	• Check the validity of the results obtained in the experiments (check all the validation conditions as described in corresponding section). • With Applied Biosystems: check if "NONE" is selected in "PASSIVE REFERENCE" field. • With LightCycler® 2.0: Check that the colour compensation file was created from Colour Compensation r-gene® (Argene ref.: 71-103) and stored in the LightCycler® 2.0 software. • Always compare the result of the extraction + inhibition control of the sample with the reference inhibition control (see section "Interpretation of results "). Dilute the sample if necessary.

ARGENE

14.2. Fluorescent signal on negative samples.

POSSIBLE CAUSES	RECOMMENDATIONS
Contamination during experiment.	• Follow all recommendations in section « Warnings and precautions ». • Decontaminate the cooling block for capillaries with U.V. light. • Respect the manufacturer's recommendations for the decontamination of automated extraction workstation and real time PCR instrument. • Samples and ENTEROVIRUS R-gene® kit must be handled only by a trained staff.
Pipetting error.	• Check the calibration of your pipettes. • Check the distributed volumes of reagents and samples: 15 µL of premix and 10 µL of sample. • Carefully homogenize reagents and samples before their distribution in capillaries/wells/tubes.
Programming error.	• Check all programming data (detection channel, mode, number of cycles, temperature and time). • Check all the steps regarding the entry of the samples.
Error in data analysis.	• Check the baseline adjustment.
Error in results interpretation.	• Check the validity of the results obtained in the experiments (check all the validation conditions described in corresponding section). • With Applied Biosystems: check if "NONE" is selected in "PASSIVE REFERENCE" field. • With LightCycler® 2.0: Check that the colour compensation file was created from Colour Compensation r-gene® (Argene ref. : 71-103) and stored in the LightCycler® 2.0 software. • Always compare the result of the extraction + inhibition control of the sample with the reference inhibition control (see section "Interpretation of results "). Dilute the sample if necessary.

14.3. The samples all seem inhibited.

POSSIBLE CAUSES	RECOMMENDATIONS
Inadequate extraction stage.	• Check that the samples have been properly homogenised before being extracted. • In the case of manual extraction using the DNA EXTRACTION KIT R-gene®. ref. : 67-000, perform the number of washes and incubation time stated in the "DNA Extraction Kit" section. • Check the materials and protocols used for extracting samples. Kit performances are only validated for the extractions described in the "Sample extraction protocol" section. • Carefully monitor to ensure that the extraction devices are maintained in line with the manufacturer's recommendations.
The IC1W0 does not result from the same extraction run	• Make sure that every sample tested includes the same batch of IC1 as IC1W0. • Each extraction run should have its own IC1W0.

ARGENE

15. Performance of the assay

WARNING: **The described performances of the kit can only be guaranteed for the recommended extraction systems and PCR instruments**

15.1. Intra-assay and inter-assay reproducibility of ENTEROVIRUS R-gene® kit

15.1.1. Intra-assay reproducibility

- The intra-experimental reproducibility studies of the ENTEROVIRUS R-gene® kit were performed on 3 positive samples.
  These samples were analysed 10 times, on a LightCycler® 2.0, following manual extraction using the " QIAamp® Viral RNA Mini kit" (QIAGEN) then diluted to obtain strong, medium and weak samples.
  The table shows the average CT obtained for each sample:

	CT Average	Standard deviation	Coefficient of variation
Cox A9 strong positive sample	24.59	0.14	0,57%
Echo 9 medium positive sample	29.93	0.45	1.50%
Polio S3 weak positive sample	31.94	0.36	1.12%

- According to the quantity of virus in the sample, the coefficient of variation varied from 0.57% to 1.50%. This coefficient of variation shows a good intra-assay reproducibility of the ENTEROVIRUS R-gene® kit.
- The same experiment was performed on the positive control (**PC9**) amplified using a Rotor-gene® 6000, LightCycler® 2.0 and Smartcycler®2.0.
  The table shows the average CT obtained for each platform:

	CT Average	Standard deviation	Coefficient of variation
Positive Control (**PC9**) on LightCycler® 2.0	21.72	0.09	0.41%
Positive Control (**PC9**) on Rotor-gene® 6000	19.05	0.30	1.57%
Positive Control (**PC9**) on Smartcycler® 2.0	22.33	0.14	0.62%

- Depending on the thermocycler used, the coefficient of variation is between 0.41% and 1,57%. These values demonstrate good inter-experimental reproducibility for the positive control (**PC9**) in the ENTEROVIRUS R-gene® kit.

15.1.2. Inter-assay reproducibility

- The inter-experimental reproducibility studies of the ENTEROVIRUS R-gene® kit were performed on 3 positive samples.
  These samples were analysed 10 times, on a LightCycler® 2.0, following manual extraction using the " DNA/RNA EXTRACTION Kit " (Argene, ref.: 67-020) then diluted to obtain strong, medium and weak samples.
- The table shows the average CT obtained for each sample:

	CT Average	Standard deviation	Coefficient of variation
Cox A9 strong positive sample	26,29	0,95	3,6%
Echo 9 medium positive sample	30,62	1,12	3,65%
Polio S3 weak positive sample	35,29	0,99	2,8%

- According to the quantity of virus in the sample, the coefficient of variation varied from 2,8 % to 3,65 %. This coefficient of variation shows a good intra-assay reproducibility of the ENTEROVIRUS R-gene® kit.

- The same experiment was performed on the positive control (**PC9**) amplified using a LightCycler® 2.0.
- The table shows the average CT obtained for each platform:

	CT Average	Standard deviation	Coefficient of variation
Positive Control (**PC9**) on LightCycler® 2.0	21,44	0,35	1,6 %

- The coefficient of variation is 1,6 %. This demonstrates good inter-assay reproducibility for the positive control (**PC9**) in the ENTEROVIRUS R-gene® kit.

15.2. Analytical sensitivity of ENTEROVIRUS R-gene® kit

- Analytical sensitivity was determined on cell lines infected with Cox A16, Cox B1 and Cox A13.
- The results obtained show a detection limit of less than 0.1 $TCID_{50}$/mL for Cox-A16 and Cox-B1, less than 0.01 $TCID_{50}$/mL for Cox-A13, less than 0.06 $TCID_{50}$/mL for Echo-18 and less than 0.2 $TCID_{50}$/mL for Echo-30.
- Analysis of the last QCMD (EV-08) Enterovirus panel gives results in accordance with the expected results for the 12 samples tested. Among these samples, sample n°7 (Cox B3) which was previously quantified at 910 cp/mL, was detected with ENTEROVIRUS R-gene® kit. Thus, the analytical sensitivity of the ENTEROVIRUS R-gene® kit is below 910 cp/mL concerning the Cox B3 serotypes.

ARGENE

15.3. Analytical specificity of ENTEROVIRUS R-gene® kit

- The specificity of ENTEROVIRUS R-gene® primers and probes were analysed against sequences in GeneBank. The specificity of the ENTEROVIRUS R-gene® primers and probes were also checked by Real Time PCR on viral cell culture and bacterial strains of the following pathogenic:
  - Human herpesviruses: HSV1, HSV2, VZV, EBV, CMV, HHV6, HHV7 and HHV8;
  - Adenovirus 12;
  - JCV and BKV;
  - *Bordetella pertussis* and *parapertussis;*
  - Infuenzavirus A and B;
  - Parainfluenzavirus 1, 2 and 3;
  - RSV;
  - Calicivirus;
  - Parvovirus B19;
  - SV40.
- None of the other viruses or bacteria were amplified with the ENTEROVIRUS R-gene® kit, which clearly proves the specificity of the assay.
- Furthermore, the specificity of recognising primers and probes selected for detection of the various enterovirus serotypes in the ENTEROVIRUS R-gene® kit was determined on 263 strains of enterovirus (clinical isolates and reference strains).
- The different strains were analysed on the ABIPRISM® 7500 platforms after automatic extraction by NucliSENS® easyMag™ (Specific A + matrix protocol).
- Results are displayed on the following table:

Enterovirus species	Serotypes	Tested strains	Detected strains
Enterovirus A	Cox A4	1	1
	Cox A6	3	3
	Cox A7	2	2
	Cox A8	1	1
	Cox A10	4	4
	Cox A14	6	6
	Cox A16	11	10
	Cox A16V	1	1
	EV71	7	7
	EV76	1	1
Enterovirus B	Cox A9	8	8
	Cox B1	9	9
	Cox B2	7	7
	Cox B3	6	6
	Cox B4	9	9
	Cox B5	7	7
	Cox B6	2	2
	E1	3	3
	E2	3	3
	E3	4	4
	E4	4	4
	E5	2	2
	E6	10	10
	E7	4	4
	E9	12	11
	E11	14	14
	E12	2	2
	E13	6	6
	E14	6	6
	E15	2	2
	E16	5	5
	E17	3	3
	E18	8	8
Enterovirus B	E19	3	3
	E20	7	7
	E21	1	1
	E24	2	2
	E25	10	10
	E26	1	1
	E27	2	2
	E29	3	3
	E30	8	8
	E31	2	2
	E32	1	1
	E33	3	3
	EV 69	1	1
	EV 74	1	1
	EV 75	2	2
	EV 77	2	2
	EV 78	1	1
	EV 93	1	1
Enterovirus C	Cox A11	1	1
	Cox A13	4	4
	Cox A17	2	2
	Cox A20	2	2
	Cox A21	3	3
	Cox A24	3	3
	Cox A24V	5	5
	EV 95	1	0
	PV1 SL	4	4
	PV2 SL	3	3
	PV3 SL	4	4
Enterovirus D	EV 68	4	4
	EV 70	1	1
	EV 94	2	2
Total	**65**	**263**	**260**

- The results obtained from the analytical specificity study show that the ENTEROVIRUS R-gene® kit can be used to detect 260 strains of the 263 tested.
- Of the 68 serotypes recognised in the 2005 international classification (*8th report of the International Committee on the Taxonomy of Viruses. 2005*), 60 were tested and detected using the ENTEROVIRUS R-gene® kit**.**
- Moreover, 2 new variants (Cox-A16V and Cox-A24V) and 3 new serotypes (EV93. EV94 and EV95) were tested, and only EV-95 was not detected by the ENTEROVIRUS R-gene® kit**.**

ARGENE

15.4. Test report from QCMD panel 2010

- During European test campaigns for Enterovirus proposed by the QCMD in 2010, 12 samples for the Enterovirus / Parechovirus panel were tested blind using the ENTEROVIRUS R-gene® kit.
- 200 µL of each sample were extracted using the manual extraction kit QIAamp® Viral RNA Mini kit (QIAGEN) and eluted at 50 µL then amplified on ABI Prism® 7500 Fast (Applied Biosystems) with the specific amplification premix R9 included in the ENTEROVIRUS R-gene® kit.

	QCMD Results				ENTEROVIRUS Virus R-gene™
	Content	Sample type*	Stock titre Dilution Sample content matrix TCID50/0.05ml	Dilution factor	CT
EV/HPeV 2010-01	Echovirus 30	Core (EV)	$2.5x10^{-5}$	$1x10^{-5}$	25.86
EV/HPeV 2010-02	Echovirus 30		$2.5x10^{-5}$	$1x10^{-7}$	39.97
EV/HPeV 2010-03	Coxasckievirus A9	Core (EV)	$3.0x10^{-6}$	$1x10^{-5}$	26.98
EV/HPeV 2010-04	Parechovirus 3		$5.6x10^{-6}$	$1x10^{-4}$	NEG
EV/HPeV 2010-05	Coxasckievirus A9	Core (EV)	$3.0x10^{-6}$	$1x10^{-7}$	41.44
EV/HPeV 2010-06	Coxasckievirus B3	Core (EV)	$5.0x10^{-6}$	$1x10^{-6}$	35.25
EV/HPeV 2010-07	EV / HPeV Negative	Core			NEG
EV/HPeV 2010-08	Enterovirus 71	Core (EV)	$1.0x10^{-5}$	$1x10^{-5}$	29.22
EV/HPeV 2010-09	Echovirus 11	Core (EV)	$2.5x10^{-7}$	$1x10^{-5}$	29.97
EV/HPeV 2010-10	Parechovirus 3		$5.6x10^{-6}$	$1x10^{-6}$	NEG
EV/HPeV 2010-11	Enterovirus 71		$1.0x10^{-5}$	$1x10^{-7}$	44.44
EV/HPeV 2010-12	Echovirus 11		$2.5x10^{-7}$	$1x10^{-7}$	41.67

- 12/12 (100%) of the samples tested are in line with the expected results.
- 9 samples of 12 were Enterovirus positive, 2 samples were Parechovirus positive and 1 sample was negative.
- The results obtained on the “Core” samples, selected by the QCMD due to the clinical relevance they represent, are 100% concordant with the expected results.
- The negative sample is confirmed negative by the kit.
- The detection of samples of low viral load for Enterovirus (EV/HPeV 2010-09 and EV/HPeV 2010-12 à 2.5.10-7) attests to the high sensitivity of the ENTEROVIRUS R-gene® kit.
- The absence of cross-reaction between Enterovirus and Parechovirus demonstrates the specificity of Enterovirus primer and probes selected for the ENTEROVIRUS R-gene® kit.

15.5. Validation of joint DNA+RNA extraction with their respective extraction tests IC1 and IC2

- To validate the possibility of producing a joint DNA/RNA extraction within the context of DNA or RNA viral pathologies (meningitis, meningoencephalitis) we produced the extraction of samples of HSV1 (DNA) and Enterovirus (RNA) with either IC2 or IC1 or a mixture of IC1 + IC2. The CT values for IC1 and IC2 obtained, are given in the following tables:

	CT	
	IC1	**IC1+IC2**
W0	26,37	26,48
Cox B2-1	26,13	26,66
Cox B2-2	26,35	26,78
Cox B2-3	26,17	26,31

	CT	
	IC2	**IC1+IC2**
W0	34,15	33,12
HSV1-1	33,68	33,27
HSV1-2	34,42	33,23
HSV1-3	33,91	33,13

- The CT values obtained are equivalent for IC1 alone or the IC1+IC2 mixture. Similarly, the values obtained are equivalent for IC2 alone and the IC1+IC2 mixture.
- This study shows that the presence of two internal tests (IC1 and IC2) does not alter the value obtained with internal tests alone. We can therefore validate the joint use of IC1 and IC2 during the extraction stage.

ARGENE

15.6. Clinical study on ENTEROVIRUS R-gene® kit

15.6.1. Clinical study on 143 samples collected in the department of "Bactériologie-Virologie-Hygiène" in "Hôpital Nord" (St Etienne, France)

- 143 samples, 78 CSF and 65 respiratory samples (taken from the throat, nasopharyngeal secretions, broncho-alveolar liquid), previously characterised using routine laboratory technique (cell culture, laboratory's own PCR technique or commercial PCR kit), were analysed by PCR in Real-time using the ENTEROVIRUS R-gene® kit on ABI PRISM® 7500, after NucliSENS® easyMag® automatic extraction with Generic and/or Specific A + matrix protocols.
  Results are displayed on the following table:

1. Analysis of all samples:

		Laboratory Technique		
		+	-	
ENTEROVIRUS R-gene®	+	99	0	99
	-	2	42	44
		101	42	143

Coefficient of concordance = 98,6%

- **Analysis of discordant results:**
  The two discordant samples were weak positives in the laboratory PCR. Note that a negative sample, concordant with both PCR techniques, was found to be positive on culture and identified as Cox-B4.

2. Analysis per type of sample:

CSF samples

		Laboratory Technique		
		+	-	
ENTEROVIRUS R-gene®	+	51	0	51
	-	0	27	27
		51	27	78

Coefficient of concordance = 100%

- No discordant was found in this type of sample.
- Note that a sample extracted simultaneously with the two "Generic" and "Specific A in the presence of matrix" protocols were perfectly extracted with the "Generic" protocol but contained inhibitors after extraction using the "Specific A" + matrix protocol.

Respiratory samples

		Laboratory Technique		
		+	-	
ENTEROVIRUS R-gene®	+	48	0	48
	-	2	15	17
		50	15	65

Coefficient of concordance = 96,92%

- **Analysis of discordant results:**
- As indicated above, the two discordant samples were detected as weakly positive using the laboratory technique. These were two throat samples. Note that extraction with the "Specific A" protocol gave a further discordant compared with "Generic" extraction.

15.6.2. Clinical study on 152 samples, collected in the National Reference Center for enteroviruses, department of Virology in "Groupement hospitalier Est" (Lyon, France)

- 152 samples, including 119 CSF, 20 respiratory samples (taken from the throat, nasopharyngeal secretions, broncho-alveolar liquid) and 13 stool samples previously characterised using the routine laboratory technique (cell culture, laboratory's own PCR technique or commercial PCR kit), were analysed by PCR in Real-time using the ENTEROVIRUS R-gene® kit on ABI PRISM® 7500, after NucliSENS® easyMag™ automatic extraction with the Specific A protocol.
  Results are displayed on the following table:

1. Analysis of all samples:

		Laboratory Technique		
		+	-	
ENTEROVIRUS R-gene®	+	104	0	104
	-	9	39	48
		113	39	152

Coefficient of concordance = 94,08%

- **Analysis of discordant results:**
  Of the nine discordant samples, 6 had been identified as weakly positive using the laboratory technique and 1 sample was inhibited.

ARGENE

2. Analysis per type of sample:

CSF samples:

		Laboratory Technique +	Laboratory Technique -	
ENTEROVIRUS R-gene®	+	74	0	74
	-	7	38	45
		81	38	119

Coefficient of concordance = 94,12%

- **Analysis of discordant results:**
  Of the seven discordant samples, 6 had been identified as weakly positive (CT> 35 cycles) using the laboratory technique and 1 sample was inhibited.

Respiratory samples

		Laboratory Technique +	Laboratory Technique -	
ENTEROVIRUS R-gene®	+	17	0	17
	-	2	1	3
		19	1	20

Coefficient of concordance = 90%

- **Analysis of discordant results:**
  The 2 discordant samples were from nasopharyngeal secretions infected with Cox-A16 and Echo-9 which were also detected by our technique (see chapter “Analytical specificity of ENTEROVIRUS R-gene® kit ").

Stool samples

		Laboratory Technique +	Laboratory Technique -	
ENTEROVIRUS R-gene™	+	13	0	13
	-	0	0	0
		13	0	13

Coefficient of concordance = 100%

CONCLUSION:

- All the results obtained during these two clinical studies demonstrate the quality of the ENTEROVIRUS R-gene® kit.
- Furthermore, within the context of using the NucliSENS® easyMag™ automatic extraction system, the performance of the ENTEROVIRUS R-gene® kit was improved by using the "Generic" protocol compared with the "Specific A + matrix" protocol.

16. References

Programming guidelines depending on device, are available on request.

- Sylvie PILLET, Geneviève BILLAUD, Shabir OMAR, Bruno LINA, Bruno POZZETTO, Isabelle SCHUFFENECKER
  Multicenter evaluation of the ENTEROVIRUS R-gene® real time RT-PCR assay for the detection of enteroviruses in clinical specimens
  Journal of Clinical Virology 2010 Jan ;47(1) :54-9
- PILLET S., BILLAUD G., OMAR S., LINA B., POZZETTO B., SCHUFFENECKER I.
  Multicenter evaluation of the ENTEROVIRUS r-gene® real time rt-pcr assay for the detection of enteroviruses in clinical specimens. ESCV, Istanbul, September 2009.
- BES J., VIGNOLES M., RAOUX N., BARTHET S., BARRANGER C., JOANNES M.
  Developpement of a new diagnostic tool for the real-time qualitative detection of ENTEROVIRUS RNA. ESCV, Istanbul, September 2009.
- BES J., MAGRO S., GROSSIORD C., VIGNOLES M., RAOUX N., BARRANGER C., JOANNES M.
  Development of a new diagnostic tool for the real time qualitative of Enterovirus RNA. EUROPIC, Barcelone, May 2008.

17. Related products

- RICO Extra r-gene®, Argene ref.: 71-105
- CELL Control r-gene®, Argene ref.: 71-106
- Colour compensation r-gene®, Argene ref.: 71-103

ARGENE

18. Index of symbols

Symbol	Meaning
	Catalogue number
	In Vitro Diagnostic Medical Device
	Manufacturer
	Temperature limitation
	Use by
	Batch code
	Consult Instructions for Use
	Contains sufficient for <n> tests
	Protect from light
	Keep dry
	Identification of notified body

BIOMERIEUX, the blue logo, ARGENE, R-gene, easyMAG and NucliSENS are used, pending and/or registered trademarks belonging to bioMérieux, or one of its subsidiaries, or one of its companies.
Any other name or trademark is the property of its respective owner.

BIOMÉRIEUX

bioMérieux SA
Chemin de l'Orme
69280 Marcy-l'Etoile - France

RCS LYON 673 620 399
Tel. 33 (0)4 78 87 20 00
Fax 33 (0)4 78 87 20 90
www.biomerieux.com